Differentiation-associated Na\(^+\)-dependent Inorganic Phosphate Cotransporter (DNPI) is a Vesicular Glutamate Transporter in Endocrine Glutamatergic Systems*

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RUNNING TITLE: *DNPI as a vesicular glutamate transporter in endocrine cells*

FOOTNOTES

The first two authors contributed equally to the present work.

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The abbreviations used are: BNPI, brain-specific Na+-dependent inorganic phosphate cotransporter; DNPI, differentiation-associated Na+-dependent inorganic phosphate cotransporter; GFAP, glial fibrillary acidic protein; MOPS, 3-N-morpholinopropanesulfonic acid; SLMVs, synaptic-like microvesicles.

NOTE-ADDED IN PROOF

Just before our submission, the following paper entitled “Molecular and functional analysis of a novel neuronal vesicular glutamate transporter” by Bai L., et al., appeared in JBC In Press on June 29, 2001. In the paper, they cloned mouse cDNA similar to DNPI. When this cDNA was expressed in PC12 cells, the vesicular glutamate transport activity appeared.
ABSTRACT

Vesicular glutamate transporter is present in neuronal synaptic vesicles and endocrine synaptic-like microvesicles, and is responsible for vesicular storage of L-glutamate. A brain-specific Na⁺-dependent inorganic phosphate transporter (BNPI) functions as a vesicular glutamate transporter in synaptic vesicles, and the expression of this BNPI defines the glutamatergic phenotype in the central nervous system (Bellocchio, E. E., et al., (2000) Science, 289, 957-960; Takamori, S., et al., (2000) Nature, 407, 189-194). However, since not all glutamatergic neurons contain BNPI, an additional transporter(s) responsible for vesicular glutamate uptake has been postulated. Here, we report that differentiation-associated Na⁺-dependent inorganic phosphate cotransporter (DNPI), an isoform of BNPI (Aihara, Y., et al., (2000) J. Neurochem. 74, 2622-2625), also transports L-glutamate at the expense of an electrochemical gradient of protons established by the vacuolar proton pump when expressed in COS7 cells. Molecular biological and immunohistochemical studies have indicated that besides in neuronal cells DNPI is preferentially expressed in mammalian pinealocytes, αTC6 cells, clonal pancreatic α cells, and α cells of Langerhans islets, these cells being proven to secrete L-glutamate through Ca²⁺-dependent regulated exocytosis followed by its vesicular storage. Pancreatic polypeptide-secreting F cells of Langerhans islets also expressed DNPI. These results constitute evidence that DNPI functions as another vesicular transporter in glutamatergic endocrine cells as well as neurons.
L-Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system, and plays important roles in many neuronal processes such as fast synaptic transmission and neuronal plasticity (1,2). To use L-glutamate as an intercellular signaling molecule, neuronal cells develop glutamatergic systems comprising the storage of glutamate in synaptic vesicles and its exocytosis (signal output), glutamate receptors (signal input), and glutamate reuptake systems (signal termination). Recent evidence has indicated that peripheral endocrine cells also develop glutamatergic systems (3,4). For instance, mammalian pinealocytes, endocrine cells for melatonin, secrete L-glutamate through Ca\textsuperscript{2+} -dependent regulated exocytosis and use it as a paracrine- or autocrine-like chemical transmitter to inhibit melatonin synthesis (3,4).

Vesicular glutamate transporter plays its primary role in the storage of L-glutamate in neurons (5,6) and endocrine cells (3,4) through the transport of L-glutamate at the expense of an electrochemical gradient of protons that is established by vacuolar H\textsuperscript{+}-ATPase. Although vesicular glutamate transporter has been characterized to some extent, its protein nature has not been known long. Very recently, brain-specific Na\textsuperscript{+}-dependent inorganic phosphate cotransporter (BNPI), representing a family of proteins that use the inwardly directed Na\textsuperscript{+} gradient across the membrane and transport inorganic phosphate (7), has been identified as the vesicular glutamate transporter in synaptic vesicles (8,9). Upon expression in either PC12 or BON6 cells, BNPI becomes associated with secretory vesicles and accumulates L-glutamate (8,9). BNPI is associated with synaptic vesicles in various glutamatergic neurons (8-11). However, it is not present in all glutamatergic neurons (8-11), suggesting that another vesicular glutamate transporter(s) may function in the neurons lacking BNPI (12).

Differentiation-associated Na\textsuperscript{+}-dependent inorganic phosphate cotransporter (DNPI), a homologue of BNPI isolated from AR42J cells differentiating into neuroendocrine cells, shows 82 % amino acid identity and 92 % similarity to human BNPI (13). In human and rat, the DNPI gene as well as the DNPI protein was shown to be expressed in neurons in various regions, especially in the encephalon, its expression patterns being somewhat different from that of BNPI (14-16). Furthermore, DNPI was shown to be located in synaptic vesicles in the neocortex (16). One can expect that DNPI is another vesicular glutamate transporter.
In the present study, we tested this hypothesis, and found that DNPI shows ATP-dependent glutamate transport activity when expressed in COS7 cells. We also showed that besides in neurons DNPI is also present in pinealocytes, αTC6 cells, and α and pancreatic polypeptide-secreting F cells in Langerhans islets, which contain a glutamatergic system.

**EXPERIMENTAL PROCEDURES**

*Organs and Cell cultures*

Pineal glands and Langethans islets were isolated from male Wistar rats at postnatal weeks 6. COS7 cells and αTC6 cells, a clonal α cell line (17), were maintained in Dulbecco's modified Eagle medium (DMEM, GIBCO) supplemented with 10% fetal calf serum, 55 µg/ml sodium pyruvate, 4.5 g/l glucose, 0.1 mg/l streptomycin, 100 units/ml penicillin G and 0.25 mg/l fungizone, and incubated at 37°C under 5% CO2. The dispersed cells were washed three times with the above medium, and then placed in a 35 mm culture dish coated with poly-L-lysine to give 2.0 x 10^5 cells/dish, and cultured in the above medium at 37°C under 5% CO2. Pinealocytes were cultured as described previously (18). For experimental procedures, cells were maintained for 5 days, washed with culture medium, cultured further for 1 h, and then used for experiments.

*Expression of DNPI*

Rat DNPI cDNA, as previously described (13), was subcloned into the EcoRI site of expression vector pcDNA3.1 (Invitrogen, San Diego, CA, USA). The resultant construct, DNPI-pcDNA3.1, was used to transfected COS7 cells by lipofection using Trans IT reagent (Mirus, Madison, WI, USA). COS7 cells were grown in DMEM containing 10% fetal calf serum, penicillin, and streptomycin in a humidified incubator at 37 °C with 5% CO2. After incubation for 24 h in 35 mm dishes, DNPI-pcDNA3.1 or the pcDNA3.1 vector alone was transfected into COS7 cells by adding 2 µg of the plasmid DNA per dish. After further incubation for 48 h, the cells were rinsed with 1 ml of buffer comprising 20 mM MOPS-Tris (pH 7.0), 0.3 M sucrose, 2 mM Mg-acetate and 4 mM KCl, and then used for further experiments.
Reverse transcription polymerase chain reaction (RT-PCR) and Northern blot analysis

Total RNA extracted from isolated glands (1 µg) was transcribed into cDNA in a final volume of 20 µl of a reaction buffer containing 0.5 mM each dNTP, 10 mM dithiothreitol, 25 pmol of random hexamers, and 200 units of Molony murine leukemia virus reverse transcriptase (Amersham). After 1 h incubation at 42°C, the reaction was terminated by heating at 90°C for 5 min. For PCR amplification, the 10-fold diluted synthesized cDNA solution was added to the reaction buffer containing 1.2 mM total dNTP (300 µM each dNTP), 6 pmole of primers and 0.5 units of PLATINUM Pfx DNA polymerase (GIBCO). Thirty-temperature cycles were conducted each cycle being as follows: denaturation at 94°C for 15 sec, annealing at temperatures specific for each set of primers for 30 sec, and extension at 68°C for 1 min. The amplification products were analyzed by polyacrylamide gel electrophoresis. The sequences of the oligonucleotides used as primers were based on published sequences (13): sense primer, 5'-AACACATCAACCAAGCAAGTC-3' (bases 2304-2324), antisense primer, 5'-AGGTAGTGAATGGGAGAGCA-3' (bases 2896-2915).

For Northern blot analysis, mRNA (4.5 µg) isolated from pineal gland or other tissues was separated on a formaldehyde agarose gel (1 %) and then transferred to a nylon membrane (Amersham). The immobilized RNA was probed with cDNA fragments of DNPI labeled with [32P]dCTP by random priming. After extensive washing, the membrane was subjected to autoradiography using BAS 1000 film (Fuji Film Co.).

L-Glutamate or D-aspartate uptake

DNPI-expressing COS7 cells were rinsed with 1 ml of buffer comprising 20 mM MOPS-Tris (pH 7.0), 0.3 M sucrose, 2 mM Mg-acetate and 4 mM KCl. The cells were then permeabilized for 10 min at 37°C in 0.5 ml of the same buffer containing 10 µM digitonin (19,20). The medium was then replaced with fresh buffer containing Tris-ATP at 2 mM in the absence of digitonin. In some experiments, bafilomycin A1 or other chemicals were also included in the medium at the specified concentrations. Then, glutamate uptake was immediately started by the addition of radioactive L-glutamate (2.5 µCi, 0.1 mM) at 37°C as described previously (20). After 10 min incubation, uptake was terminated by washing the cells twice with 1 ml of ice-cold 20 mM MOPS-Tris (pH 7.0) containing 0.3 M sucrose.
the cells were lysed with 1 ml of 1 % SDS, and the radioactivity and protein concentration were measured. In some experiments, radiolactive D-aspartate (2.5 µCi, 0.1 mM) was used for the substrate.

**Antibodies**

Site-specific polyclonal antibodies against rat DNPI were raised in rabbits using synthetic polypeptides corresponding to the C-terminal 12 residues, C-DAYSYKDRDDYS (GenBank accession number, 8515880; protein ID number, AAF76223.1). The polypeptide was conjugated with hemocyanine, keyhole limpet (Calbiochem), with m-maleimidebenzoyl-N-hydrosuccinimide ester. The monoclonal antibodies against synaptophysin and vimentin (VIM3B4) were obtained from Progen. The monoclonal antibodies against glial fibrillary acidic protein (GFAP) and OX42 were purchased from Reo Markers and Cosmo Bio, respectively. The mouse monoclonal antibodies against EEA1 and rab 5, for early endosomes, and GM130, for the cis Golgi apparatus, were obtained from Transduction Laboratories. Monoclonal antibodies against PDI for endoplasmic reticulum were from Fuji Yakuhin Kogyo Co. Ltd. (Toyama, Japan). The monoclonal antibodies against glucagon and insulin (MAB1) were from Sigma and Cymbus Biotechnology Ltd., respectively. The rat monoclonal antibodies against somatostatin were from Chemicon. Guinea pig polyclonal antisera against rat pancreatic polypeptide were from Linco Research, Inc.

**Immunoblotting**

Membrane fractions (particulate fractions) of rat brain, pineal gland, Langerhans islets and cultured cells prepared as described (18, 20) were denatured with SDS sample buffer containing 1 % SDS and 10 % β-mercaptoethanol, and then electrophoresed on 12 % polyacrylamide gel in the presence of SDS. Following electrotransfer at 0.3 amperes for 2 h, the nitrocellulose filters were blocked in a buffer consisting of 20 mM Tris-Cl (pH 7.6), 5 mM EDTA, 0.1 M NaCl and 0.5 % bovine serum albumin for 4 h, and then probed with 1000-diluted anti-DNPI antibodies in the same buffer. The filters were washed with 20 mM Tris-Cl buffer (pH 7.6) containing 5 mM EDTA, 0.1 M NaCl and 0.1 % Tween 20, treated with peroxidase-labeled anti-rabbit IgG or anti-mouse IgG at a dilution of 1:2000 for 30 min, washed further with the same buffer, and then subjected to ECL amplification according to the
manufacturer's manual (Amersham).

**Immunohistochemistry**

The published procedure was used (21,22). In brief, cells on poly L-lysine-coated glass coverslips were fixed in 4% paraformaldehyde for 20 min, washed with phosphate-buffered saline, incubated with the same buffer containing 0.1% Triton X-100 for 30 min, then further with 2% goat serum and 0.5% bovine serum albumin in the same buffer, and finally reacted with antibodies at the specified dilution as described in the figure legends in phosphate-buffered saline containing 0.5% bovine serum albumin for 1 h at room temperature. The samples were washed three times with phosphate-buffered saline and then reacted with the second antibodies for 1 h at room temperature. The second antibodies used were AlexaFluor 568-labeled anti-mouse IgG at 1 µg/ml, AlexaFluor 488-labeled anti-rabbit IgG at 2 µg/ml, Cy3-labeled goat anti-rat IgG at 1 µg/ml, Cy3-labeled anti-rabbit IgG at 2 µg/ml or FITC-labeled anti-guinea pig IgG at 2 µg/ml. These second antibodies were obtained from Amersham or Molecular Probes. Finally, immunoreactivity was examined under an Olympus Fluoview FV300 confocal laser microscope.

**Other procedures and chemicals**

DNA sequencing was performed by the chain-termination method (23). Protein concentrations were determined with a Pierce Protein Assay Kit with bovine serum albumin as a standard. L-[2,3-^3^H]-glutamate (9.25 MBq) and D-[2,3-^3^H]-aspartate (9.25 MBq) were obtained from NEN Life Science Products, Inc. (Boston, USA). Digitonin was purchased from Wako Chemical Co. (Osaka, Japan). Other chemicals were of the highest grade commercially available.

**RESULTS**

**DNPI as a Vesicular Glutamate Transporter**

To examine whether or not DNPI is vesicular glutamate transporter, DNPI was expressed in COS7 cells. As shown in Fig. 1A, anti-DNPI antibodies recognized a major broad protein band corresponding to an apparent molecular mass of ~65 kDa when DNPI-
pcDNA3.1, a DNPI expressing vector, was transfected to COS7 cells. The molecular mass corresponding to the DNPI immunoreactivity is similar to that expected from its primary amino acid sequence and DNPI from the brain (Fig. 1A). Two additional protein bands with an apparent molecular mass of ~72 and ~42 kDa were also observed. Neither untransfected control COS7 cells nor COS cells transfected with a control vector expressed any DNPI gene, as revealed on RT-PCR analysis (data not shown), or the immunoreactive polypeptide (Fig. 1A). The DNPI immunoreactivity disappeared when the antigenic polypeptide was included during antibody treatment (Fig. 1A). These results indicated that DNPI is expressed in COS7 cells.

The expressed DNPI distributed throughout COS7 cells (Fig. 1B). Immunohistochemical analysis indicated that DNPI is co-localized with EEA1 or rab 5, early endosomal markers (Fig. 1C). DNPI is partially co-localized with GM130, a marker protein of the cis Golgi apparatus, but not with PDI, a marker protein of endoplasmic reticulum (Fig. 1C). These results suggest that DNPI is mainly associated with early endosomes in the cells.

We examined whether or not DNPI shows vesicular glutamate transport activity. As shown in Fig. 1D, digitonin-permeabilized DNPI-expressing cells took up radiolabeled L-glutamate depending on ATP. Neither untransfected control cells nor cells transfected with a control vector showed ATP-dependent glutamate uptake activity. The omission of Mg$^{2+}$ reduced the ATP-dependent L-glutamate uptake to the control level. Bafilomycin A1, a specific inhibitor of vacuolar H$^+$-ATPase (24), at 1 µM inhibited the ATP-dependent L-glutamate uptake. SF6847, a proton conductor that dissipates an electrochemical proton gradient, also inhibited the ATP-dependent L-glutamate uptake. In contrast, vanadate (1 mM), an inhibitor of P-type ion-transporting ATPases, did not affect the ATP-dependent glutamate uptake. These results indicated that the glutamate uptake is driven by an electrochemical gradient of protons established by vacuolar H$^+$-ATPase. The addition of either L-aspartate or D-aspartate at 1.0 mM during the assay had a little effect on the ATP-dependent glutamate uptake, indicating that the glutamate transporter does not recognize L-aspartate or D-aspartate as a substrate, one of the characteristics of vesicular glutamate transporters (25-27). Inability that DNPI transports D-aspartate was also confirmed by the direct uptake assay (Fig. 1D).
Together, these results indicate that DNPI is responsible for the vesicular storage of L-glutamate.

**DNPI is co-localized with SLMVs in pinealocytes**

Besides the central nervous system, peripheral endocrine tissues possess glutamatergic systems (3,4). Mammalian pinealocytes accumulate L-glutamate in SLMVs, counterparts of synaptic vesicles in endocrine cells, and secreted it to the extracellular space through exocytosis (28,29). Vesicular glutamate transporter is responsible for the storage of L-glutamate in pineal SLMVs (27,30). To determine whether or not DNPI is expressed in pinealocytes, expression of the DNPI gene in pineal gland was examined by RT-PCR using specific DNA probes. As shown in Fig. 2A, amplified products of expected size for DNPI were obtained when total RNAs isolated from pineal glands and cultured pineal cells as well as brain were used. The nucleotide and deduced amino acid sequences of the amplified products exactly matched that of the DNPI gene. Northern blot analysis with the amplified RT-PCR products further demonstrated the expression of mRNA for DNPI in pineal glands: two major bands (~3.2 and 4.1 kb) for pineal glands and brain mRNA were detected (Fig. 2B). Western blot analysis indicated that the anti-DNPI antibodies recognized a single polypeptide of ~ 65 kDa in pineal glands, cultured pinealocytes and brain membranes (Fig. 2C). The DNPI immunoreactivity was blocked when the nitrocellulose sheet was treated with an antigenic peptide during the immunodecoration (Fig 2C). Overall, it is concluded that DNPI is expressed in pineal glands and cultured pinealocytes.

Immunohistochemistry with frozen-sectioned pineal gland revealed the localization of DNPI at the cellular level. We used the following cell markers to classify DNPI-positive cells: synaptophysin for pinealocytes (30,31), GFAP for astrocytes (32), OX-42 for microglia (33), and vimentin for interstitial cells (34). The antibodies against these marker proteins immunostained the corresponding populations of pineal cells with a similar morphology, as reported previously (33) (Fig. 2D). The DNPI-positive cells coincided with synaptophysin, but not with any of the above mentioned cell markers, indicating that pinealocytes contain DNPI (Fig. 2D). Essentially the same results were obtained for cultured pineal cells (data not shown). DNPI and synaptophysin are enriched in the process terminal regions, the site
for glutamate exocytosis (3,4) (Fig. 2E). These results strongly suggested that DNPI is associated with SLMVs in pinealocytes.

**DNPI in α cells of Langerhans islets**

Langerhans islets express various types of ionotropic glutamate receptors and reuptake systems (36-38). Clonal pancreatic αTC6 cells store and secrete L-glutamate through exocytosis, the mechanism being similar to those in neurons and pinealocytes (20). Thus, it is possible that Langerhans islets are another example of an endocrine glutamatergic system and that DNPI is responsible for the storage of L-glutamate in the islets. To examine this possibility, the expression of DNPI in αTC6 cells and islets was measured. RT-PCR analysis indicated the presence of DNPI mRNA in αTC6 cells (Fig. 3A). Western blotting and immunohistochemistry indicated the presence of DNPI in αTC6 cells (Fig. 3B,C).

Langerhans islets are composed of four major types of endocrine cells, i.e. insulin-secreting β cells, glucagon-secreting α cells, pancreatic polypeptide (PP)-secreting F cells and somatostatin-secreting δ cells. Western blotting clearly indicated the presence of DNPI in the islets (Fig. 3B). DNPI was co-localized with glucagon but not with insulin or somatostatin in horizontal sections of the islets, indicating the presence of DNPI in α cells but not in β or δ cells (Fig. 3C). DNPI is also co-localized with pancreatic polypeptides. These results suggested that DNPI is mainly present in α cells and partially in pancreatic polypeptide-secreting F cells (Fig. 3C).

**DISCUSSION**

Vesicular glutamate transporter is responsible for the glutamatergic characteristics of neurons and was originally identified in synapsin I-associated synaptic vesicles (25,39). In the earlier stage of studies, vesicular glutamate transporter was defined as the ATP-dependent proton conductor-sensitive glutamate transport activity in synaptic vesicles, but little was known about the protein nature of the transporter at the molecular level (25-27, 39). In the last year, two groups have independently reported that BNPI is vesicular glutamate transporter itself, and that BNPI is a potential tool for substantial studies on vesicular
glutamate transporter. DNPI is a potential candidate for another vesicular glutamate transporter, since DNPI is distributed throughout the brain, being especially abundant in the nerve endings of glutamatergic neurons, where BNPI is scarce (15,16). Here we showed that DNPI actually functions as a vesicular glutamate transporter when expressed in COS7 cells.

DNPI expressed in COS7 cells seems to be mainly localized in endosomes. Since endosomes contain vacuolar H\(^+\)-ATPase (40, 41), the active transport of glutamate into the organelles should be expected upon the addition of ATP in digitonin-permeabilized cells. In fact, digitonin-permeabilized cells took up L-glutamate depending on MgATP and the properties of the uptake are consistent with those of vesicular glutamate transporter (25-27, 39), indicating that DNPI expressed functions as a vesicular glutamate transporter.

The fact that DNPI exhibits vesicular glutamate transport activity is not surprising, since the amino acid identity of the core portions of DNPI and BNPI excluding their hydrophilic N- and C-terminal regions are over 90% with 12 putative transmembrane helices (7,13). In the original studies on the expression and functions of BNPI and DNPI, both proteins were found to facilitate the transport of inorganic phosphate depending on extracellular Na\(^+\) (7,13). On the other hand, vesicular glutamate transporters use a proton as a coupling ion, and only recognize L-glutamate and a few cyclic glutamate analogues as substrates (25-27, 39). Thus, DNPI and BNPI are versatile in their coupling ions and substrate specificity in nature. Further studies will be necessary to elucidate the molecular mechanism underlying the versatility, and to assign the domains responsible for the Na\(^+\)-dependent inorganic phosphate transport and proton-coupled glutamate transport.

Significant finding in this study is that DNPI is expressed in glutamatergic endocrine cells. Recent studies have revealed that some endocrine cells secrete L-glutamate through exocytosis, and the released glutamate may function as a paracrine- or autocrine chemical transmitter by way of a glutamate receptor expressed on the same or neighboring cells (3, 4). Vesicular glutamate transporters play a key role in glutamate signal output through the storage of L-glutamate in endocrine cells. Consistent with the presence of glutamatergic systems, DNPI is present in pinealocytes and is co-localized with SLMVs. Thus, DNPI may
function to store L-glutamate in SLMVs and secrete it through exocytosis, which is one of the components of the negative regulatory mechanism for melatonin synthesis. Not all pinealocytes processes are positive for DNPI (Fig. 2E, arrows and arrowheads), indicating the functional heterogeneity of SLMVs in pinealocytes, as suggested by Redecker (42).

It should be emphasized that DNPI is present in αTC6 cells and α cells in Langerhans islets. DNPI is also present in pancreatic polypeptide-secreting F cells, suggesting that pancreatic polypeptide-secreting F cells are capable of storing and secreting L-glutamate. Therefore, α cells and possibly pancreatic peptide-secreting F cells are the sites for the glutamate release in the islets, which was first proposed by Weaver et al., (38). Thus, Langerhans islets may equip their own input, output and termination systems for glutamate signals. Although the physiological role(s) of the glutamatergic system in the islets is not fully understood at present, the glutamate may regulate the secretion of insulin and glucagon by way of its binding to the receptors in α and β cells (43,44). A role of L-glutamate as an intracellular signaling molecule in β cells, which enhances the second phase of insulin secretion, has also been postulated (45).

In conclusion, DNPI is an indicator for glutamatergic systems in peripheral tissues as well as neurons, and is a very useful probe for studies on peripheral glutamatergic systems. We are extensively studying the localization of DNPI in various organs, which will reveal the site where L-glutamate acts as an intercellular signaling molecule in peripheral organs.
REFERENCES

272, 257-265.


FIGURE LEGENDS

Figure 1.  DNPI expressed in COS7 cells functions as a vesicular glutamate transporter.
A. Expression of DNPI in COS7 cells. Membrane fractions prepared from brain (lanes 1 and 4), control (vector alone) cells (lane 2), and DNPI-pcDNA3.1 transfected cells (lanes 3 and 5) (100 µg protein) were solubilized with SDS sample buffer and then subjected to SDS polyacrylamide gel electrophoresis followed by immunoblotting using anti-DNPI antibodies. The immunoreactivity was visualized with ECL. For lanes 4 and 5, the nitrocellulose sheet was incubated with 1 mg antigenic peptide during the antibody treatment. The positions of the molecular markers are shown. B. Immunohistochemical detection of DNPI expressed in COS7 cells. Control (vector alone) cells and DNPI-pcDNA3.1 transfected cells were immunostained with anti-DNPI antibodies (x 1000) and then observed under a fluorescence microscope. Bar = 20 µm. C. Subcellular localization of DNPI expressed in COS7 cells was investigated. DNPI-pcDNA3.1 transfected cells were doubly immunostained with antibodies against (1) DNPI (green) and EAA1 (red), (2) DNPI (green) and rab 5 (red), (3) DNPI (green) and GM130 (red), or (4) DNPI (green) and PDI (red), and then observed under a confocal microscope. Dilution of the antibodies is as follows: EEA1 x 100, rab 5 x 100, GM130 x 200 and PDI x 50. The superposition of the two images was also shown. Bar = 10 µm. D. The ATP-dependent uptake of L-glutamate by digitonin-permeabilized DNPI-pcDNA3.1 transfected cells. Glutamate uptake by permeabilized cells was monitored as described under EXPERIMENTAL PROCEDURES in the presence or absence of the listed compounds; 1 µM bafilomycin A1, 0.5 µM SF6847, 1.0 mM sodium vanadate, 1.0 mM L-aspartate, and 1.0 mM D-aspartate. The ATP-dependent D-aspartate uptake was also measured (lower panel). In some experiments, Mg-acetate (-Mg²⁺) or ATP (-ATP) was omitted. Control (vector alone) cells or untransfected cells were also permeabilized with digitonin and their glutamate uptakes under the standard condition were measured. The results are the means ± SEM of four independent experiments.

Figure 2. Expression and localization of DNPI in pinealocytes.
A. RT-PCR detection of gene expression of DNPI in pineal gland and cultured cells.
Transcript of DNPI for brain (lanes 2 and 5), pineal gland (lanes 3 and 6), and cultured pineal cells (lanes 4 and 7). The PCR product was not detected if reverse transcriptase was omitted from the reaction mixture (lanes 5 - 7). The apparent molecular mass is also shown (lane 1).

B. Expression of mRNA for DNPI was measured by Northern blotting. The amplified PCR products were hybridized with total RNA from brain (lane 1), pineal gland (lane 2), liver (lane 3), or PC12 cells (lane 4) and the resultant hybridization was visualized with a BAS2000. The positions of 18S and 28S RNA were shown. The lower panel shows expression of G3PDH as a control.

C. DNPI protein was detected by Western blotting. Membrane fractions prepared from pineal gland (lanes 1 and 4), cultured pineal cells (lanes 2 and 5) (100 µg protein) and brain (lanes 3 and 6) (50 µg protein) were solubilized, electrophoresed, and then subjected to Western blotting with anti-DNPI antibodies as described in the legend to Fig. 1A. For lanes 4 - 6, the nitrocellulose sheet was incubated with 1 mg of antigenic peptide during the antibody treatment. The positions of the molecular markers were shown.

D. Immunohistochemical localization of DNPI in pineal gland. Sections of a pineal gland were doubly immunostained with antibodies against (1) DNPI (green) and synaptophysin (red), (2) DNPI (green) and GFAP (red), (3) DNPI (green) and OX-42 (red), or (4) DNPI (green) and vimentin (red), and then observed under a confocal microscope. The superposition of the two images was also shown. Bar = 10 µm.

E. Immunohistochemical localization of DNPI in cultured pinealocytes. Cultured pinealocytes were doubly immunostained with antibodies against DNPI (green) and synaptophysin (red), and then observed under a confocal microscope. Arrows indicated process terminal that contains DNPI and synaptophysin. Arrowheads indicated the process terminal lacking DNPI. Dilution of the antibodies is as follows: DNPI x 1000, synaptophysin x 50, OX42 x 800, GFAP x 100, and vimentin x 10. Bar = 10 µm.

**Figure 3.** DNPI is expressed in αTC6 cells and Langerhans islets.

A. RT-PCR detection of gene expression of DNPI in αTC6 cells. Transcripts of DNPI for cultured αTC6 cells (lane 2). The PCR product was not detected if reverse transcriptase was omitted from the reaction mixture (lane 3). The apparent molecular mass is also shown (lane 1).

B. DNPI was detected on Western blotting. Membrane fractions prepared from αTC6...
cells (lanes 1 and 3) and Langerhans islets (lanes 2 and 4) (50 µg protein each) were solubilized, electrophoresed, and subjected to Western blotting with anti-DNPI antibodies as described in the legends of Fig. 1A. For lanes 3 and 4, the nitrocellulose sheet was incubated with 1 mg of antigenic peptide during the antibody treatment. The positions of the molecular markers were shown.

C. Immunohistochemical localization of DNPI. (1) DNPI is particularly abundant in αTC6 cells. Sections of Langerhans islets were doubly immunostained with antibodies against (2) DNPI (green) and glucagon (red), (3) DNPI (green) and insulin (red), (4) DNPI (green) and somatostatin (red), or (5) DNPI (red) and pancreatic polypeptide (PP) (green), and then observed under a confocal microscope. The superposition of the two images was also shown. Dilution of the antibodies is as follows: DNPI x 1000, glucagon x 50,000, insulin x 200, somatostatin x 200, and pancreatic polypeptide (PP) x 1000. Bar = 10 µm.
Fig. 1A
Fig. 1B

vector

DNPI-pcDNA3.1
Fig. 1C
Fig. 1D

Glutamate uptake (nmol/1x10^5 cells)

- Complete (+ ATP)
- -ATP
- +Bafilomycin A1 (1 μM)
- +SF6847 (500 nM)
- -Mg^{2+}
- L-Aspartate (1 mM)
- D-Aspartate (1 mM)
- +Vanadate (1 mM)
- Untransfected cell
- Control cell (Vector alone)

D-Aspartate uptake (nmol/1x10^5 cells)

- Complete (+ ATP)
- -ATP
- Control cell (Vector alone)
Fig. 2A
Fig. 2B
Fig. 2 C
Fig. 2E
Fig. 3A
Fig. 3B
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